EXHIBIT 8

Ovalbumin is synthesized in mouse cells transformed with the natural chicken ovalbumin gene*

(recombinant DNA/eukaryotic gene transfer/gene expression in transformed cells/solid-phase radioimmunoassay)

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ABSTRACT   The entire chicken ovalbumin gene, accompanied by genomic DNA sequences flanking both termini of the gene and three copies of the herpes simplex virus thymidine kinase gene, has been cloned in plasmid pBR322. This recombinant plasmid was linearized and used to transform thymidine kinase-deficient mouse cells. Thymidine kinase-positive transformants were selected by their ability to grow in the hypoxanthine/aminopterin/thymidine (HAT) medium. The entire ovalbumin gene integrated into high molecular weight DNA within all the transformants and retained its original sequence organization. In all of the transformants examined, a protein identified as chicken ovalbumin by immunoreactivity was detected within the cells. It is estimated that between 1000 and 100,000 molecules of chicken ovalbumin were produced per mouse cell in each of these transformants. Our results demonstrate that the mouse cellular machinery can be utilized to accurately express genetic information encoded in a cloned gene from a different eukaryotic organism into its specific protein product.

The cloning of the entire ovalbumin gene from the chicken genome has allowed us and others to establish the detailed molecular structure of this hormone-regulated gene (1–3). This well-defined gene can now be used to study the mechanism by which steroid hormones regulate its expression. One approach is to examine the functional capacity of the cloned ovalbumin gene under intracellular conditions after reintroduction into a eukaryotic cell. Cultured mammalian cells transformed with DNA coding for selectable biochemical markers have been found to express these gene functions (4–6). A 3.4-kilobase (kb) DNA fragment containing the thymidine kinase (TK) gene from herpes simplex virus type I (HSV) has been cloned (7) and used to transform mutant mouse cells deficient in thymidine kinase (LMTK⁻), resulting in the appearance of colonies that were of the TK⁺ phenotype (6). The transfer into mouse LMTK⁻ cells of a gene that does not code for a selectable marker has been effected by cotransformation with the TK gene (8, 9). We report here the transformation of mouse LMTK⁻ cells with the chromosomal ovalbumin gene by using this DNA-mediated gene transfer system. The ovalbumin polypeptide has been detected in all of the TK⁺ transformants containing the complete ovalbumin gene.

MATERIALS AND METHODS

Cell Culture. Two strains of the mouse LMTK⁻ cells originally derived by Kit *et al.* (10) were used for these studies. One tetraploid line (Cl 1D) has been described (11). The second cell line (LTK⁻ APRT⁻) is an adenine phosphoribosyltransferase-deficient (APRT⁻) derivative of LMTK⁻ cells generously

provided to us by R. Axel, Columbia University. Cells were maintained in Dulbecco's modified Eagle's medium with high glucose (GIBCO) supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA), penicillin at 100 units/ml, and streptomycin at 100 μg/ml (GIBCO). After transformation, cell cultures were maintained in the above growth medium containing hypoxanthine at 15 μg/ml, aminopterin at 0.2 μg/ml, and thymidine at 5.0 μg/ml (HAT).

Transformation and Selection. Cells were seeded at a density of 6×10^5 per 100-mm dish for 24 hr, rinsed with Hanks' balanced salt solution, and inoculated with DNA according to Wigler *et al.* (4). DNA was diluted to a final concentration of 20 μg/ml in Hepes-buffered saline (135 mM NaCl/5 mM KCl/0.7 mM Na$_2$HPO$_4$/0.11% dextrose/20 mM Hepes, pH 7.1). The mixture was brought to 125 mM CaCl$_2$, allowed to stand at room temperature for 30 min, and spread onto the rinsed cells (0.5 ml per dish). After 30 min at room temperature, 10 ml of Dulbecco's modified Eagle's medium plus 5% fetal calf serum was added and the cells were incubated at 37°C for 40 hr. The cells were then maintained in the HAT selective medium, which was changed every 3 days. After 20 days, individual HAT-resistant colonies were cloned by using the cylinder technique (12). Each clone was propagated in culture dishes for four or five passages before culturing in 850-cm² roller bottles (Falcon).

Containment. All recombinant DNA experiments were performed in P2 and P3 facilities in accordance with National Institutes of Health guidelines.

Analysis of DNA from Transformed Cells. Transformed cells were lysed directly in the roller bottle by the addition of 20 ml of a buffer containing 2% sodium dodecyl sulfate, 7 M urea, 0.35 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0. The cell lysate was extracted with an equal volume of buffer-saturated phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol). Nucleic acid in the aqueous fraction was precipitated by ethanol. After treatment with RNase (50 μg/ml), the DNA was recovered by phenol extraction and ethanol precipitation. Agarose gel electrophoresis of restriction endonuclease-digested DNA and Southern blotting experiments were performed as described (13).

Ovalbumin Immunoassay. A solid-phase radioimmunoassay described by Broome and Gilbert (14) was used to detect ovalbumin production in TK⁺ mouse cells. The cells were pelleted and lysed by three freeze-thaw cycles. A 1-μl sample of lysate was spotted onto a 1.3% agarose gel matrix in phos-

Abbreviations: TK, thymidine kinase; HSV, herpes simplex virus type 1; HAT, hypoxanthine/aminopterin/thymidine; kb, kilobase.
* This paper is no. 13 in a series of publications from our laboratory dealing with the structure, organization, function, and regulation of the ovalbumin gene in the chicken oviduct. Paper no. 12 is ref. 2.

phate-buffered saline. The gel was placed in contact at 4°C for several hours with a polyvinyl sheet that had been coated with anti-ovalbumin IgG. The polyvinyl sheet was lifted from the gel and incubated overnight at 4°C with ^{125}I-labeled anti-ovalbumin IgG (^{125}I-IgG). The sheet was washed, dried, and autoradiographed by using a Kodak XR-5 film and a Du Pont Cronex Hi-plus intensifying screen at −70°C. The assay could detect picogram amounts of chicken ovalbumin. Polyacryl-amide gradient (5–20%) slab gel electrophoresis of mouse cell extracts was performed in a buffer consisting of 40 mM Tris·HCl, pH 7.8, 5 mM Na acetate, 0.1 mM EDTA. Bands con-taining immunoreactive ovalbumin were again visualized by an adaption of the solid-phase radioimmunoassay. The gel was overlaid with an anti-ovalbumin IgG-coated polyvinyl sheet at 4°C for 3 hr. The sheet was then incubated with ^{125}I-IgG and the bands were visualized by autoradiography.

RESULTS

pOV12 is a chimeric plasmid containing the entire ovalbumin gene with 3.5 and 0.5 kb of DNA flanking the 5′ and 3′ termini of the gene, respectively. This recombinant plasmid (Fig. 1) was constructed by partial *Hin*dIII digestion of a λ recombinant isolated from a chicken gene library (2, 15). The 12 kb of chicken DNA was inserted into the *Hin*dIII site of plasmid pBR322. The orientation of the ovalbumin gene insert is such that the unique *Bam*HI site in the plasmid is located at about 1 kb from the 3′ end of the gene (Fig. 1). Because the 12 kb chicken DNA fragment is not cleaved by *Bam*HI, pOV12 was linearized with this enzyme and the 3.4-kb *Bam*HI fragment of HSV DNA containing the TK gene was introduced into the cleavage site. The resultant chimeric plasmid contains three copies of the TK fragment and is designated pOV12-TK (Fig. 1). *Hin*dIII cleaves pOV12-TK at four sites, excising the entire

chicken DNA insert out of pBR322 in three fragments 4.8, 4.0, and 3.2 kb in length. *Hha* I cleaves pBR322 DNA at many po-sitions, but does not cut the chicken DNA. This enzyme digests pOV12-TK DNA into many small fragments and one large fragment of 12.2 kb consisting of the entire chicken DNA insert and about 0.2 kb of plasmid sequence. *Sal* I cleaves the plasmid sequence once but does not cut the chicken DNA and the TK DNA inserts.

pOV12-TK was linearized by *Sal* I (Fig. 1) and was used to transform mutant mouse LMTK⁻ cells. A total of 21 TK⁺ transformants was obtained from three dishes (Table 1). Six of these clones, representing cells from all three dishes, were cultured and expanded to about 4 × 10⁷ cells in roller bottles. High molecular weight DNA prepared from the transformants was cleaved by *Hha* I, followed by Southern blotting using a ³²P-labeled ovalbumin DNA probe. All six transformants showed a predominant hybridization signal at 12.2 kb, indi-cating that the entire 12 kb of chicken DNA from pOV12-TK was present within single stretches of cellular DNA (Fig. 2A, lanes b–g). Several weaker hybridization signals of various sizes were observed in addition to the 12.2-kb band, indicating that multiple copies of the chicken DNA of different lengths might be present in each of the transformed cell lines. *Hin*dIII digests of DNA from transformed cells revealed three common hy-bridization bands at 4.8, 4.0, and 3.2 kb as expected (Fig. 2B, lanes b–g). Digestion of the DNA by *Eco*RI and *Pst* I also re-vealed all the characteristic hybridization signals of the chicken DNA (data not shown). These results demonstrated that the chicken DNA had retained its original sequence organization within the mouse cells. Furthermore, the ovalbumin gene is present in high molecular weight DNA (>50 kb) as analyzed by Southern blotting using uncut DNA from the transformed mouse cells (data not shown), suggesting that the gene has been integrated into cellular DNA.
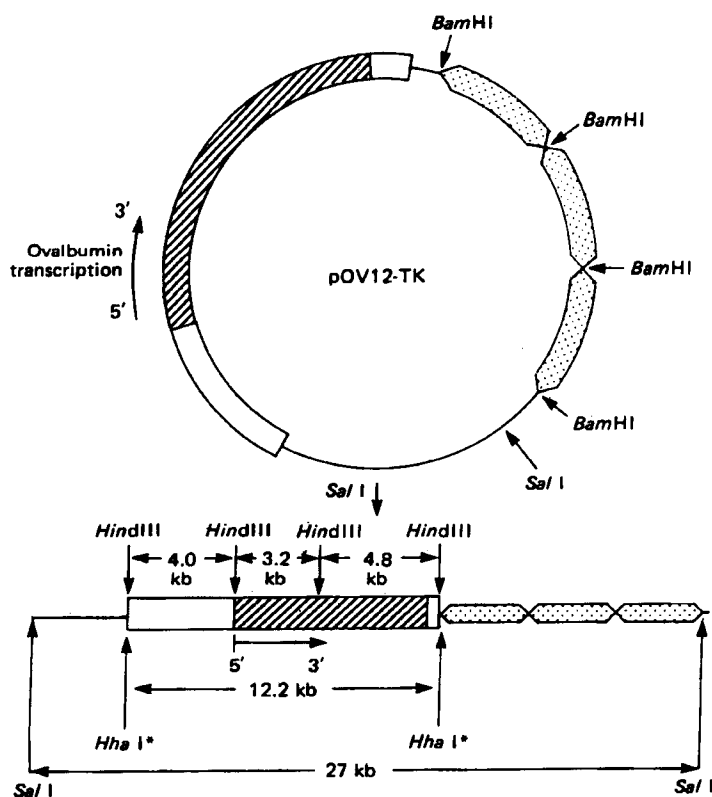

FIG. 1. Restriction endonuclease map of pOV12-TK. Chimeric plasmid pOV12-TK consists of pBR322 with a 12-kb chicken DNA fragment containing the entire ovalbumin gene cloned in its *Hin*dIII site and three copies of a 3.4-kb HSV DNA fragment containing the TK gene inserted in its *Bam*HI site. The chimeric plasmid is 27 kb in size and was linearized by *Sal* I digestion before being used for transformation experiments. The chicken ovalbumin gene is represented by hatched bars, flanking chicken DNA by empty bars, HSV DNA by stippled bars, and pBR322 by a line.
* *Hha* I does not cleave the chicken DNA in pOV12-TK, and *Hha* I* represents the cleavage sites for this enzyme in pBR322 DNA immediately flanking the chicken DNA insert. Other *Hha* I sites in pBR322 and HSV TK DNA are not shown.

Table 1.   Transformation experiments with the chicken ovalbumin gene*

Source of DNA	DNA per dish, µg			TK⁺ clones obtained†	Clones analyzed	Clones containing the complete ovalbumin gene	Clones producing ovalbumin
	Chicken	HSV TK	Salmon sperm carrier				
Sal I-digested pOV12-TK	0.65	0.55	8.5	21	6	6	6
BamHI-digested pOV12 ligated in vitro to BamHI fragment of HSV TK DNA‡	6.9	0.65	—	14	7	1	1
BamHI-digested pOV12	7.3	—	—	0	—	—	—
Sal I-digested pTK	—	0.5	8.9	39	—	—	—

* All dishes were overlaid with 10 µg of DNA in 0.5 ml of the DNA/calcium phosphate mixture. The amount of plasmid DNA per dish is not shown in this table.

† These are the total number of clones obtained from three dishes.

‡ pOV12 was linearized by BamHI digestion and ligated to the 3.4-kb BamHI fragment of HSV TK DNA (molar ratio 3:1) at a final DNA concentration of 500 µg/ml and using phage T4 DNA ligase (Bethesda Research Laboratories, Rockville, MD) at 30 units/ml.

A separate transformation experiment was carried out to test the efficiency of DNA-mediated gene transfer using a different method of target DNA preparation. The chimeric plasmid pOV12 was linearized by BamHI digestion and ligated in vitro to the 3.4-kb BamHI DNA fragment containing the TK gene. This concatenated DNA preparation was used to transform mouse LTK⁻ cells as described for the previous experiment. A total of 14 TK⁺ clones was obtained from three petri dishes (Table 1). Only 3 out of 7 of the clones analyzed contained portions of the ovalbumin gene. One of the transformants (mOV12-A) possessed the three expected HindIII fragments of 4.8, 4.0, and 3.2 kb (Fig. 2B, lane h). Additional weaker hybridization signals were also detected in the same DNA sample and may represent the presence of multiple copies of the chicken DNA in this transformed clone. Thus, only 1 out of 7 such transformants contained the entire 12-kb chicken DNA fragment from pOV12.

Having established that the chicken ovalbumin gene was present in the transformed TK⁺ clones, we next examined whether the gene could be expressed in the mouse cell. A sensitive solid-phase screening method of in situ immunoassay was employed to detect ovalbumin from the cell extracts of individual TK⁺ clones. Picogram amounts of antigen could be detected in this assay (Fig. 3). Lanes h, i, and j indicate the signals generated by 10, 5, and 2.5 pg of crystalline egg-white ovalbumin, respectively. There were no detectable amounts of ovalbumin in the host LMTK⁻ cells (Fig. 3, lane k). One-microliter samples of extracts from individual clones were analyzed in the same assay, and all of these clones were found to contain significant quantities of immunoreactive ovalbumin (lanes a–g). In clone mOV12-4, 300 pg of ovalbumin was present in about 5×10^4 cells. It is estimated that approximately 100,000 molecules of ovalbumin polypeptide were present per cell. In addition, only one of the transformants (mOV12-A) generated from the chicken and TK DNA ligated in vitro synthesized ovalbumin (lane g).

In order to further characterize the ovalbumin synthesized in various mouse clones, the cell extracts were analyzed by polyacrylamide gel electrophoresis followed by in situ radioimmunoassay for ovalbumin (Fig. 4). Lane a contained 500 pg of crystalline egg-white ovalbumin. The protein separated into two distinct molecular species on the gel due to differing levels of phosphorylation. Cell extracts from all three ovalbumin-producing mouse clones also showed two immunoreactive bands after electrophoresis (Fig. 4, lanes b–d), although they did not migrate exactly as the ovalbumin standards did. This difference in migration should not be interpreted as a consequence of differing peptide length because the gel electrophoresis could not be performed under denaturing conditions. Rather, the small differences in electrophoretic mobility probably represent dissimilar or incomplete posttranslational modifications of the polypeptides.
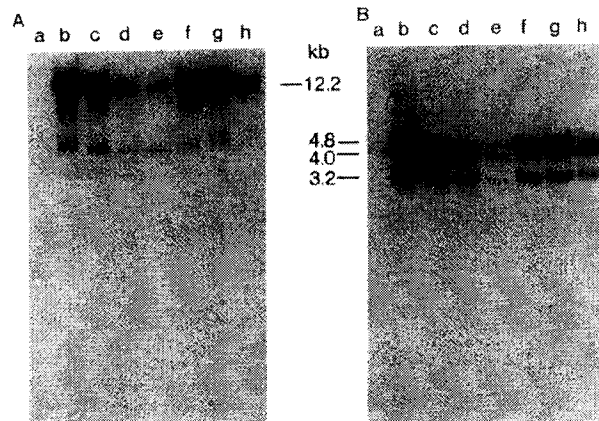

FIG. 2.   Autoradiograph of a 1% agarose gel containing DNA from individual TK⁺ transformants after digestion with restriction endonuclease and Southern hybridization. Twelve micrograms of mouse cellular DNA from various TK⁺ clones was digested with Hha I (A) or HindIII (B). After electrophoresis, the DNA was transferred to a nitrocellulose filter and Southern hybridization was carried out with ³²P-labeled OV12 DNA. OV12 DNA free of plasmid sequences was prepared by preparative agarose gel electrophoresis after digestion of pOV12 DNA with HindIII and Hha I. HindIII released the chicken DNA insert from pOV12 as three fragments 4.8, 4.0, and 3.2 kb in length, whereas Hha I cut the plasmid sequences into multiple fragments less than 0.4 kb. Lanes a, host LMTK⁻; lanes b, mOV12-1; lanes c, mOV12-3; lanes d, mOV12-4; lanes e, mOV12-8; lanes f, mOV12-10; lanes g, mOV12-2; lanes h, mOV12-A.
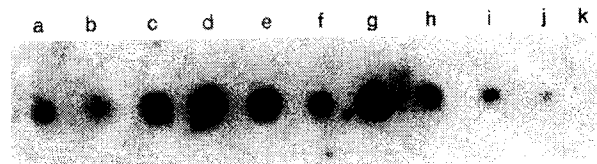

FIG. 3.   Autoradiograph of a solid-phase immunoassay for ovalbumin in cell extracts of TK⁺ transformants. Samples (1 µl) of the cell lysates from different TK⁺ transformant clones were analyzed. Lanes h, i, and j indicate signals generated by 10, 5, and 2.5 pg of crystalline egg-white ovalbumin, respectively. Lane k contains extract from host LMTK⁻ cells and lanes a, b, c, d, e, f, and g contain cell extracts from clones mOV12-1, mOV12-2, mOV12-3, mOV12-4, mOV12-8, mOV12-10, and mOV12-A, respectively.
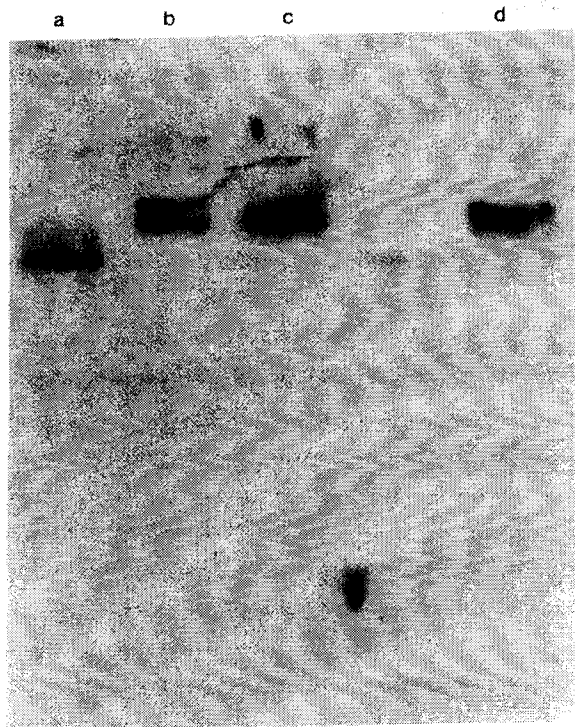
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a   b   c   d

FIG. 4. Autoradiograph of 5–20% polyacrylamide gradient gel electrophoresis of cell lysates to detect chicken ovalbumin. Electrophoresis was performed at 132 V for 4 hr. Lane a, chicken ovalbumin; lane b, clone mOV12-4; lane c, clone mOV12-8; lane d, clone mOV12-A.

DISCUSSION

We have transformed mouse Cl 1D cells with pOV12-TK DNA, which is a chimeric plasmid containing the complete normal chicken ovalbumin gene and the HSV TK gene. In all of the TK$^+$ transformants examined, the entire 12-kb chicken DNA was found to have been integrated into high molecular weight cellular DNA and to have retained its original sequence organization. Thus, large foreign DNA fragments not coding for any selectable biochemical marker can be used to transform mouse LMTK$^-$ cells with high efficiency if first cloned with the HSV TK gene. It was also possible to transform mouse LTK$^-$ cells with foreign DNA that was ligated to the HSV TK gene *in vitro*. However, the efficiency of obtaining transformants containing the entire ovalbumin gene was greatly reduced in our hands and appeared to depend largely on the ligation conditions employed. Three out of seven TK$^+$ transformants obtained from such experiments contained some chicken DNA sequences and only one of them possessed the entire 12-kb chicken DNA fragment. When this cell line (mOV12-A) was allowed to grow in medium without HAT for three generations and then transferred back to a HAT medium, no decrease in plating efficiency was observed. Furthermore, when BrdUrd was added to the growth medium at 30 μg/ml, more than 99.8% of the mOV12-A cells died within 2 days of culture (data not shown). These preliminary results suggest that the presence of the TK gene in this cell line is quite stable. Further experiments are needed to determine the fate of the ovalbumin gene within these cells.

A protein identified as chicken ovalbumin by immunoreactivity was detected from the cell lysates of several of the transformant mouse clones. The ovalbumin polypeptide must therefore be synthesized in cells transformed with the ovalbu-

min gene. When total protein extracted from transformed cells was subjected to electrophoresis and analyzed for ovalbumin by radioimmunoassay, two distinct protein bands were obtained. However, they migrated slightly slower than those of crystalline egg-white ovalbumin standards (Fig. 4). It should be noted that ovalbumin secreted from chicken oviduct cells is a complex protein containing an NH$_2$-terminal acetyl group, carbohydrate residues, and two phosphate residues. Because the gel electrophoresis was not performed under denaturing conditions, the observed discrepancy in mobility for ovalbumin produced by the mouse cells versus egg-white ovalbumin is not necessarily a reflection of chain length but may be due to a slightly dissimilar or incomplete mode of posttranslational modification. Further studies are necessary to distinguish the precise physical characteristics of these proteins.

This slight discrepancy of electrophoretic mobility of the proteins notwithstanding, the production of a protein recognizable by an antibody against ovalbumin in the mouse cells transformed by the natural ovalbumin gene is indicative of correct expression of this gene in a heterologous cell, although preliminary analysis has shown that not all ovalbumin RNA molecules are perfect transcripts of the ovalbumin gene. The chromosomal chicken ovalbumin gene is in excess of 7.5 kb in length, coding for a mature ovalbumin messenger RNA of less than 2000 nucleotides with the difference made up by seven intervening sequences (2). The entire ovalbumin gene appears to be transcribed in the oviduct cells into a 7.8-kb precursor RNA, which is subsequently processed into the mature mRNA through splicing (16). In order to produce a correct protein in a mouse cell transformant, not only must the ovalbumin gene be transcribed correctly but also the precursor RNA must be properly spliced. Imprecise splicing at a single nucleotide at any of the 14 structural–intervening sequence junctions of the gene would have resulted in such an aberrant protein that recognition by a specific antibody against ovalbumin would be unlikely.

The fact that the chicken ovalbumin gene transcripts could be correctly spliced in mouse L cells is not unexpected in view of the similarity between the junction sequences in the ovalbumin gene and those of the mouse β-globin gene (17, 18). It also appears that splicing occurs correctly in other heterogeneous systems such as the simian virus 40 late gene transcripts in *Xenopus* oocytes (19). By using the same DNA-mediated gene transfer system, a cloned chromosomal rabbit DNA fragment containing the β-globin gene was ligated *in vitro* to the HSV TK gene and the concatenated DNA preparation was employed for transformation of LMTK$^-$ cells (8, 9). Mature rabbit β-globin mRNA was present at various levels in the transformed mouse cells, which was indicative of proper transcription and splicing (8). However, the β-globin protein has not yet been detected in these transformed cells (8). In another DNA-mediated gene transfer system, the cloned chromosomal mouse β-globin gene was introduced into monkey kidney cells with a defective simian virus 40 DNA vector and the mouse DNA was replicated within host cells as part of the viral genome (20). Although the mouse gene was expressed at high frequency into mature β-globin mRNA and mouse β-globin, transcription appeared to have initiated at the simian virus 40 late promotor region rather than the β-globin gene promotor (20). Whether transcription of the chicken ovalbumin gene in transformed mouse cells initiated accurately at the ovalbumin gene promotor region has not been defined. Nevertheless, the expression of the ovalbumin gene in these cells should not be under the control of the TK genes because these latter genes were cloned at the 3' end of the ovalbumin gene so that read-through from TK gene transcription would have only resulted in the synthesis of the complementary strand of

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ovalbumin mRNA. In addition, the 5′ end of the ovalbumin gene was not physically linked to the TK genes because pOV12-TK had been cleaved by *Sal* I prior to transformation (Fig. 1).

At present, it is unclear why the ovalbumin gene-containing mouse transformants were synthesizing different levels of ovalbumin. Because the complete ovalbumin gene appeared to have integrated into high molecular weight cellular DNA, we conclude that the site of integration might play an important role in determining the first level of gene expression. Finally, our observation that the ovalbumin gene can be expressed properly in the mouse cells transformed with the ovalbumin gene and the fact that all of the transformants produced ovalbumin makes this DNA-mediated gene transfer system appropriate for future studies designed to elucidate the mechanism of expression of eukaryotic genes.

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